1 **Title**

2 Characterization of lignin-derived products from various lignocellulosics as treated by

3 semi-flow hot-compressed water

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22   **Abstract**

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24   To elucidate the decomposition behaviors of lignin from the different taxonomic groups,

25   five different lignocellulosics were treated by hot-compressed water (230°C/10MPa/15

26   min) to fractionate the water-soluble portion, precipitates and insoluble residues. The

27   lignin-derived products in each fraction were, then, characterized and compared. As a

28   result, the delignification from monocotyledons such as nipa palm (*Nypa fruticans*) frond,

29   rice (*Oryza sativa*) straw and corn (*Zea mays*) cob were more extensive compared to that

30   from woods such as Japanese cedar (*Cryptomeria japonica*) and Japanese beech (*Fagus*

31   *crenata*), being gymnosperm and dicotyledon in angiosperm, respectively. The water-

32   soluble portion contained lignin monomers like coniferyl alcohol and phenolic acids,

33   while the precipitates were found to consist of higher molecular weight lignin with high

34   ether type linkages. In contrast, the lignin in the insoluble residues was rich in condensed

35   type structures. Therefore, in all five lignocellulosics, ether type linkages were

36   preferentially cleaved and condensed type lignin had resistance to hot-compressed water.

37   Furthermore, regarding monocotyledons, the lignin-carbohydrate complexes were

38   cleaved and the higher molecular weight lignin were eluted compared to woods. These

39   differences would facilitate the delignification in monocotyledons. Such information

40   provides a clue as to efficient utilization of various lignocellulosics.

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42   (193 words)

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1. Introduction

Due to the depletion of fossil fuel and the environmental issues of global warming, the utilization of lignocellulosics as renewable resources has been examined towards the biofuel and biochemical production. The cell wall of lignocellulosics consists mainly of cellulose, hemicelluloses and lignin, and these components enforce a sophisticated structure, having a resistance to water and organic solvents.

For the conversion of the lignocellulosics into value-added chemicals, a variety of different decomposition methods of lignocellulosics have been explored. Hydrothermal treatments have a high hydrolysis ability and dissolve even the hydrophobic lignin because of the highly ionic products and low dielectric constant of water [1, 2]. Thus, various kinds of hydrothermal treatments of lignocellulosics have been explored [3, 4], and among hydrothermal treatments, a hot-compressed water treatment was conducted under relatively milder condition, but its potential for lignocellulosics decomposition was still quite high [5]. Such a hot-compressed water treatment can be categorized into batch, flow and semi-flow types. Among them, semi-flow system can minimize degradations because of the rapid removal of the degraded products from the reaction vessel [6, 7].

With such an advantage, our research group developed a semi-flow hot-compressed water treatment, and achieved the high yields of saccharides from cellulose and hemicelluloses [8–10]. With respect to the lignin, its decomposition behaviors of woods such as Japanese cedar and Japanese beech were discussed through the characterization of their lignin-derived products as treated by hot-compressed water [11,

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66   12].

67   In contrast, non-woody lignocellulosics such as palm, rice and corn are also

68   expected as renewable resources and they are categorized as monocotyledons in

69   angiosperms, while woods such as Japanese cedar and Japanese beech as one of

70   gymnosperms and dicotyledons in angiosperms, respectively [13]. Among these

71   lignocellulosics, there are several differences existing in the lignin structures, for instance,

72   the lignin component unit structures and the association with phenolic acids [14].

73   Accordingly, their decomposition behaviors of lignin would be different one another.

74   Thus, in this study, five lignocellulosics, which are categorized into different taxonomical

75   groups, were treated with semi-flow hot-compressed water (230°C/10MPa). To facilitate

76   comparisons on the lignin decomposition behaviors, the characterization of the lignin-

77   derived products were conducted and compared among five lignocellulosics.

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80   **2. Materials and methods**

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82   2.1 Samples and chemicals

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84   The sapwood of Japanese cedar (*Cryptomeria japonica*) as one of gymnosperms, the

85   sapwood of Japanese beech (*Fagus crenata*) as one of dicotyledons in angiosperms, the

86   nipa palm (*Nypa fruticans*) frond, the rice (*Oryza sativa*) straw and the corn (*Zea mays*)

87   cob as monocotyledons in angiosperms were selected in this study. Flour of each

88   lignocellulosics passing through an 18-mesh screen was extracted with acetone using a

89   Soxhlet apparatus and dried at 105°C for 24 h before the experiments. All chemicals used

90   in this study were of reagent grade without any purification.

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93   2.2 Characterization of lignocellulosics

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95   The chemical composition in each lignocellulosics was evaluated using the method of

96   Rabemanolontsoa et al. [15].

97   The contents of the phenolic acids, which are attached to lignin in

98   monocotyledons, were determined by the alkali extraction with 0.5M NaOH aqueous

99   solution [16]. The extracted portion was acidified and extracted with ethyl acetate. The

100   ethyl acetate-soluble portion was then dehydrated and evaporated under vacuum. The

101   obtained products were trimethylsilyl derivatized followed by gas chromatography-mass

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102 spectrometry (GC-MS: GCMS-QP2010 Ultra, Shimadzu Co., Kyoto, Japan) analysis.

103 The capillary column was a CP Sil 8CB (Agilent Technology Inc., California, United

104 States). The temperature program was 1.0 min at 40°C, 5.0°C/min to 300°C, and 8.0 min

105 at 300°C. Helium carrier gas was used at a flow-rate of 1.5 mL/min. Injector and detector

106 temperatures were both 230°C.

107   For the analysis of lignin structure, the alkaline nitrobenzene oxidation was

108 performed according to the ordinary method [17] and the total yields of vanillin,

109 syringaldehyde, and *p*-hydroxybenzaldehyde were determined by GC (GC 2014,

110 Shimadzu Co., Kyoto, Japan). The vanillin can be produced from ferulic acid, and the *p*-

111 hydroxybenzaldehyde can be from *p*-coumaric acid and 4-hydroxybenzoic acid. Thus, the

112 yields of phenolic acids-derived vanillin and *p*-hydroxybenzaldehyde were subtracted

113 from the original yields of the alkaline nitrobenzene oxidation products to obtain the

114 actual yields of products only from the core lignin.

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117 2.3 Hot-compressed water treatment

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119 Approximately 0.5 g of oven-dried extractive-free lignocellulosics was placed in the

120 reaction vessel for which the hot-compressed water treatment was performed as described

121 in a previous paper [18]. The treatment condition was 230°C/10 MPa for 15 min, in which

122 most of the hemicelluloses and part of lignin can be decomposed [18].

123   After the treatment, the hot-compressed water-insoluble residue left over in the

124   reaction vessel was separated from the hot-compressed water-soluble portion, and dried

125   at 105°C for 12 h to measure its oven-dried weight. The separated hot-compressed water-

126   soluble portion was kept standing for 12 h under ambient condition. During this period,

127   it was separated to be water-soluble portion and precipitates which were retrieved by

128   filtration with a Millipore membrane filter (pore size: 0.45 μm). For the water-soluble

129   portion, the lignin-derived products were obtained by extraction with ethyl acetate.

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132   2.4 Analytical methods of lignin-derived products

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134   The lignin-derived products in three fractions, water-soluble portion, precipitates and

135   insoluble residue, were characterized. The content of lignin-derived products in each

136   fraction was examined as the combined yields of Klason lignin and acid-soluble lignin

137   [19]. Molecular weight distribution of lignin-derived products in the water-soluble

138   portion and precipitates was evaluated by gel permeation chromatography (GPC) analysis,

139   which was performed with LC-10A (Shimadzu, Kyoto, Japan) as described in the

140   previous study [12]. Before the GPC analysis, the precipitates were acetylated [20]. The

141   alkaline nitrobenzene oxidation analysis was conducted for the precipitates and insoluble

142   residues by means of the same method described above. Monomeric lignin-derived

143   compounds in water-soluble portion were determined and quantified by GC-MS analysis,

144   which was performed with same equipment and same methods as described above.

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3. Results and discussion

3.1 Characterization of lignocellulosics

Fig. 1 shows the chemical composition of five different lignocellulosics used in this study. As for Japanese cedar, one of the gymnosperm softwoods, the cellulose content is the highest, and the lignin is secondly the highest to be 32.0wt%, followed by hemicellulose with others such as protein, extractives and ash. For Japanese beech, one of the hardwoods (dicotyledon in angiosperms), the chemical composition is relatively similar to that of Japanese cedar, however the lignin is relatively lower in content. In contrast, regarding nipa frond, rice straw and corn cob, monocotyledons in angiosperms, the lignin is lower to be around 18~20wt%, with the protein and ash being much higher compared with woods (Japanese cedar and Japanese beech).

The contents of phenolic acids, which are attached to lignin in monocotyledons, were determined as given in Table 1. For the Japanese cedar and Japanese beech, phenolic acids were not detected. In contrast, regarding the monocotyledons, the ferulic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid were obtained. The ferulic acid is known to connect hemicelluloses with lignin, known as lignin-carbohydrate complex (LCC) in the cell wall of monocotyledons [21]. In particular, the corn cob contained the highest amount of ferulic acid to be 0.77wt% on lignocellulosics-basis (= 4.3wt% on lignin-basis). For the *p*-coumaric acid, it was reported that *p*-coumaric acid is associated to the lignin of monocotyledons [22]. In a similar manner to the ferulic acid, the corn cob contained the

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168   highest amount of *p*-coumaric acid to be 1.5wt% on lignocellulosics-basis (= 8.3wt% on

169   lignin-basis). Furthermore, the nipa frond contained a high amount of *p*-hydroxybenzoic

170   acid, which was 0.78wt% on lignocellulosics-basis (= 4.0wt% on lignin-basis). In the

171   palm species, it is reported that the *p*-hydroxybenzoic acid is associated with lignin [23].

172   In addition, to examine the lignin component units in these lignocellulosics, the

173   alkaline nitrobenzene oxidation was conducted. As the decomposed products, vanillin

174   (VA) derived from the guaiacyl (G) lignin, syringaldehyde (SA) from the syringyl (S)

175   lignin and *p*-hydroxybenzaldehyde (HA) from the *p*-hydroxyphenyl (H) lignin were

176   obtained. Although the detailed yields of each product will be discussed later in Fig. 5,

177   the molar ratio of decomposed products to VA is shown in Table 1.

178   For Japanese cedar, VA was the main product with a slight amount of HA,

179   indicating, as well known, that lignin in Japanese cedar is mainly composed of G lignin

180   with a small amount of H lignin. In case of Japanese beech, on the other hand, both VA

181   and SA were resulted and the ratio of SA to VA was quite high. Regarding three

182   monocotyledons, all of VA, SA and HA were obtained. The nipa frond had a high ratio of

183   SA to VA and a quite low of HA to VA. In contrast, the rice straw and corn cob had a high

184   ratio of HA to VA.

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187   3.2 Yields of lignin-derived products in each fraction

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189   Fig. 2 shows the yields of the water-soluble portion, the precipitates and the insoluble

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190   residue for five different lignocellulosics as treated by semi-flow hot-compressed water.

191   After treatment, the hot-compressed water-soluble portion was kept standing for 12 h

192   under ambient condition, during which it was separated to be the water-soluble portion

193   and the precipitates. In case of Japanese cedar and Japanese beech, the yields of insoluble

194   residues are relatively higher and the yields of hot-compressed water-soluble portion

195   (water-soluble portion + precipitates) are low. In contrast, from monocotyledons, around

196   70wt% was decomposed and solubilized into hot-compressed water. The precipitates

197   obtained were 5~10wt% on lignocellulosics-basis for all five lignocellulosics.

198   Fig. 3 shows the yields of lignin-derived products in water-soluble portion,

199   precipitates and insoluble residue from five lignocellulosics as treated by hot-compressed

200   water. For Japanese cedar, 43wt% of lignin was decomposed and eluted in hot-

201   compressed water with more than half of lignin remaining as insoluble residue. For

202   Japanese beech, more than half of lignin was decomposed and eluted in hot-compressed

203   water, indicating that the delignification is preferentially occurred from Japanese beech

204   compared to Japanese cedar. In contrast, the delignification from monocotyledons was

205   much more preferentially occurred to be around 80wt%. Significant differences were

206   observed between the delignification from woods (Japanese cedar and Japanese beech)

207   and monocotyledons. Among the hot-compressed water-soluble lignin, 20~30wt% of

208   lignin was retrieved as the precipitated lignin.

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211   3.3 Structural analysis of lignin-derived products

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213   Fig. 4 demonstrates GPC chromatograms of water-soluble portion and precipitates from

214   five lignocellulosics, detected at a wavelength of 280 nm. In order to evaluate the

215   molecular weight distribution of their lignin-derived products, the elution times of

216   polystyrene standards (MW: 162, 580, 1270, 5000) were displayed. The higher molecular

217   weight standards show the shorter elution time in GPC chromatograms.

218   As a result, the lignin-derived products in water-soluble portion contain

219   monomeric and oligomeric products up to the molecular weight of 1,000~2,000. In

220   contrast, the molecular weight of the lignin in the precipitates are higher than those in

221   water-soluble portion for all lignocellulosics. Among five different lignocellulosics, the

222   lignin-derived products in the precipitates from monocotyledons start to elute 2~3 min

223   faster than those from woods, indicating that the lignin-derived products from

224   monocotyledons are composed of higher molecular weight products than those from

225   woods.

226   In order to compare the relative proportion of ether linkages of lignin-derived

227   products, the alkaline nitrobenzene oxidation was conducted, to achieve its yields as

228   shown in Fig. 5. In case of Japanese cedar, the yields of vanillin (VA) from lignin in the

229   precipitates are higher than those from original wood flour. This result indicates that the

230   lignin in the precipitates maintains ether linkages. For the insoluble residue, the yield of

231   VA is relatively low, thus the lignin in the insoluble residue is rich in condensed type. For

232   Japanese beech, both VA and syringaldehyde (SA) are obtained. The total yields from

233   precipitates are quite high and those from insoluble residue are low in a similar manner

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234 to Japanese cedar. Regarding the SA/VA ratio, the precipitates (2.6) is higher than the

235 original wood flour (2.2) and the insoluble reside is much lower (1.3). This could indicate

236 the precipitated lignin is rich in S lignin, but the residual lignin is in G lignin. As for the

237 monocotyledons, all of VA, SA and *p*-hydroxybenzaldehyde (HA) are obtained from all

238 fractions. The total yields from precipitates are almost similar to those from the original

239 wood flour, while the total yields from insoluble residues are lower as in woods.

240 Regarding the ratio of VA, SA and HA, the main products from the precipitates are SA

241 and the yields of VA and HA are relatively low. In contrast, as to the insoluble residues,

242 the ratio of VA and HA are relatively high.

243   Consequently, for all lignocellulosics, the lignin-derived products in precipitates

244 maintained the ether type linkages, whereas those in the insoluble residue were rich in

245 condensed type lignin. Except for Japanese cedar, being composed of only G lignin, the

246 lignin component units of precipitates contained more methoxyl group compared to those

247 of insoluble residues.

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250 3.4 Lignin-derived products in water-soluble portion

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252 From the GPC analysis, it was found that the lignin-derived products in water-soluble

253 portion contained monomers (Fig. 4). Thus, GC-MS analysis was performed to identify

254 the lignin-derived monomers in the water-soluble portion, and the yields of main products

255 was summarized in Table 2. These compounds were identified and quantified by

256   comparing the retention time and the mass fragmentation patterns with their model

257   compounds. As a result, the coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol,

258   which are the monomer precursors of lignification, were the main monomeric products.

259   The obtained monomers corresponded well to the lignin component units detected by the

260   alkaline nitrobenzene oxidation (Table 1). As for the woods, vanillin and syringaldehyde

261   were also obtained, while those aldehydes were not detected from monocotyledons.

262   In the water-soluble portion from monocotyledons, the phenolic acids were

263   obtained. Given that these phenolic acids were not obtained from woods, the obtained

264   acids would not be derived from the core lignin but from the phenolic acids attached to

265   lignin. In particular, the *p*-coumaric acid and *p*-hydroxybenzoc acid were obtained with

266   quite high yields.

267   With respect to the *p*-coumaric acid in corn cob, it was reported by 2D-NMR

268   analysis for the corn stover that the *p*-coumaric acid is attached to the lignin at γ position

269   of propane side-chain [22]. Although the detailed structures of *p*-coumaric acid in the

270   corn cob are not clarified yet, it seems to be quite possible that the high yield of *p*-

271   coumaric acid would be attached to the end position of lignin. Such a linkage would be

272   readily cleaved and resulted in a quite high yield.

273   With regards to the *p*-hydroxybenzoic acid, it is reported to be attached to the

274   lignin at γ position of its sidechain for the frond of oil palm (*Elaeis guineensis*) [23].

275   Therefore, in case of nipa palm frond, it seems quite possible that *p*-hydroxybenzoic acid

276   would attach to the end position of lignin.

277   The ferulic acid was also found from monocotyledons. The majority of the ferulic

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278   acid is known to be both esterified and etherified to lignin and hemicellulose [24, 25], to

279   form the lignin-carbohydrates complex (LCC) [21]. Thus, it would be probable that

280   ferulic acid obtained in the water-soluble portion would be from LCC linkages cleaved

281   during hot-compressed water treatment.

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284   3.5 Lignin-derived products in precipitates

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286   For all five different lignocellulosics, the lignin-derived products in the precipitates are

287   relatively higher in molecular weights, and their structures rich in ether linkages from the

288   analysis of the alkaline nitrobenzene oxidation. Regarding the precipitated lignin from

289   Japanese beech, their structures were quite similar to the milled wood lignin, considered

290   to be alike native lignin. Thus, the precipitated lignin seems to maintain the original lignin

291   structures [18]. Yamauchi et al. speculated for its reason that some micropores are

292   produced in the cell wall because of the decomposition of hemicelluloses and the cleavage

293   of lignin ether linkages at the beginning of hot-compressed water treatments. The

294   produced micropores would have then facilitated the elution of the lignin cluster, in which

295   ether type linkages are maintained to some extent [11]. Hydrophobic lignin could be

296   eluted in hot-compressed water due to the low dielectric constant of hot-compressed water,

297   whereas some large molecules cluster precipitated after hot-compressed water returned to

298   the ordinary water under ambient condition. The similar phenomena would be occurred

299   in the monocotyledons, because the lignin-derived products in the precipitates from

300   monocotyledons were composed of products with relatively higher molecular weight than

301   those from woods.

302   The ferulic acid contents of precipitates were quite low to be less than 0.1wt% on

303   precipitates basis. This indicates that the LCC structures in the cell walls would be mostly

304   cleaved during the hot-compressed water treatment. Under this condition, both

305   hemicelluloses and lignin in the cell wall were decomposed and eluted out from the cell

306   wall into hot-compressed water [8, 9]. To verify this behavior, further experiments on

307   their LCC should be performed.

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310   3.6 Lignin-derived products in insoluble residue

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312   About half of lignin from woods and about 20wt% of lignin from monocotyledons were

313   recovered as the insoluble residue. For all five different lignocellulosics, the lignin-

314   derived products were rich in condensed type lignin. Given that the ether type linkages

315   are easily cleaved by hot-compressed water treatment, but that the condensed type

316   linkages are difficult to be cleaved [26], the condensed lignin had a resistance to hot-

317   compressed water and the insoluble residues were rich in condensed type linkages.

318   Furthermore, for all lignocellulosics, the lignin in the insoluble residues were composed

319   of lower methoxyl components units compared to that in the precipitates.

320   Considering that the phenylpropane units with lower methoxyl content have

321   higher possibility to form condensed type linkages at 3 or 5 position of aromatic ring,

322 these results are reasonable. Thus, in terms of the component units, H lignin is thought to

323 be the most tolerant structure. However, the corn cob and the rice straw, which are

324 composed of high ratio of H lignin (Table 1), showed the extensive delignification

325 compared to other lignocellulosics, indicating that the decomposition of lignin would be

326 affected not only by the lignin component units but also by other lignin structures like

327 LCCs as described above.

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330 **4. Concluding remarks**

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332 Various lignocellulosics, belonging to the different taxonomic groups, were treated with

333 hot-compressed water (230°C/10MPa/15 min), and the obtained lignin-derived products

334 in each fraction were characterized. As a result, the delignification from monocotyledons

335 such as nipa palm frond, rice straw and corn cob was more extensive than that from woods

336 such as Japanese cedar and Japanese beech. For all lignocellulosics, the lignin of insoluble

337 residues were rich in condensed linkages, indicating that the decomposition of lignin due

338 to the cleavage of ether linkages. The residual lignin consisted of lower methoxyl content

339 in phenylpropane units, compared to those in the precipitates. Considering that the lignin

340 with lower methoxyl content can readily have condensed type linkages at 3 and/or 5

341 position of aromatic ring, the lower methoxyl content units reveal resistance to hot-

342 compressed water, remaining as insoluble residue. It is interesting, however, that the corn

343 cob and rice straw, which consisted of high ratio of H lignin, showed the extensive

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344   delignification compared to other lignocellulosics. Regarding the lignin-derive products

345   from monocotyledons, the LCC were cleaved and higher molecular weight lignin was

346   eluted into hot-compressed water, compared to that from the woods. These differences

347   would have facilitated the delignification from monocotyledons. Based on these lines of

348   evidence, it was found that there are several differences on the delignification and the

349   decomposition behaviors of lignin between the woods (gymnosperm softwood and

350   angiosperm dicotyledonous hardwood) and monocotyledons as treated by hot-

351   compressed water.

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354   **Acknowledgements**

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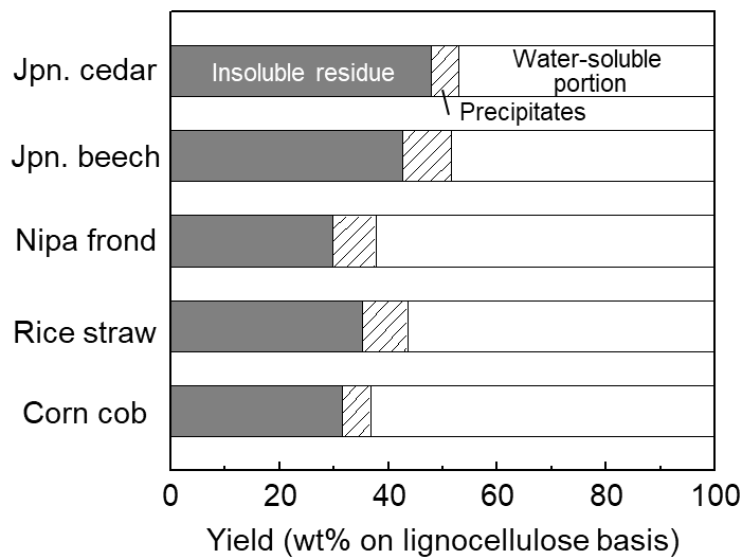424   products from wood as treated in supercritical water. J Wood Sci 48:320–325

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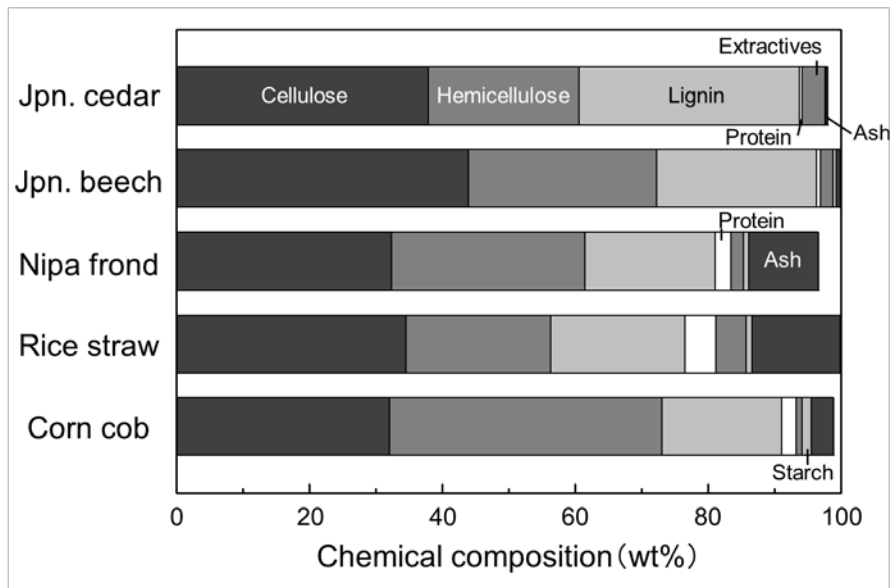
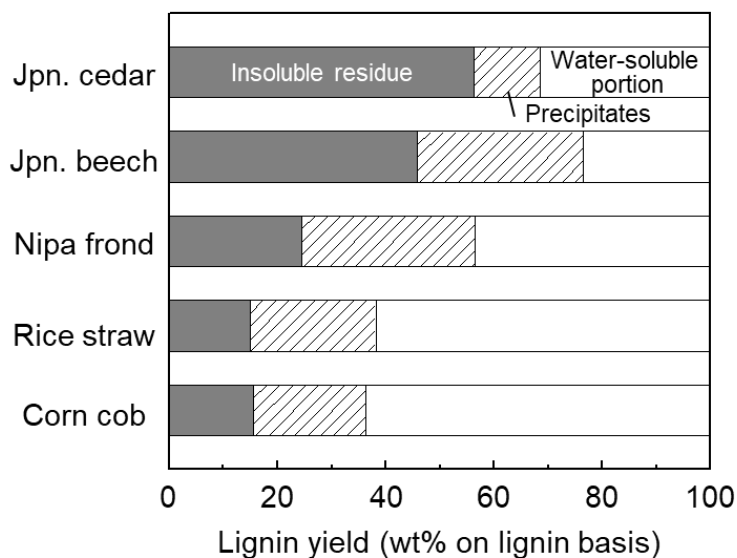Fig. 1 Chemical composition of lignocellulosics as determined by the method [13]

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Fig. 2 Yields of water-soluble portion, precipitates and insoluble residue from five different

lignocellulosics as treated by semi-flow hot-compressed water

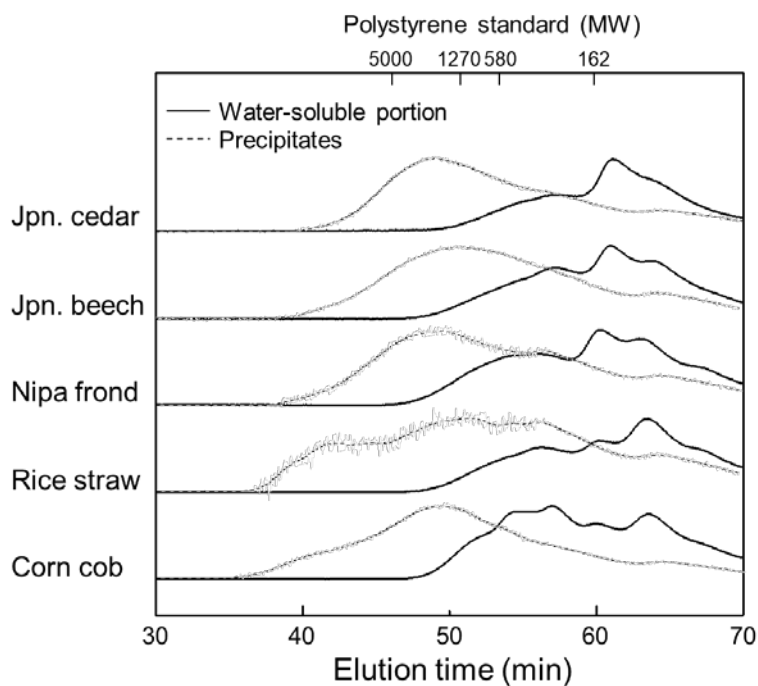434

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Fig. 3 Yields of lignin in water-soluble portion, precipitates and insoluble residue from five
different lignocellulosics as treated by semi-flow hot-compressed water

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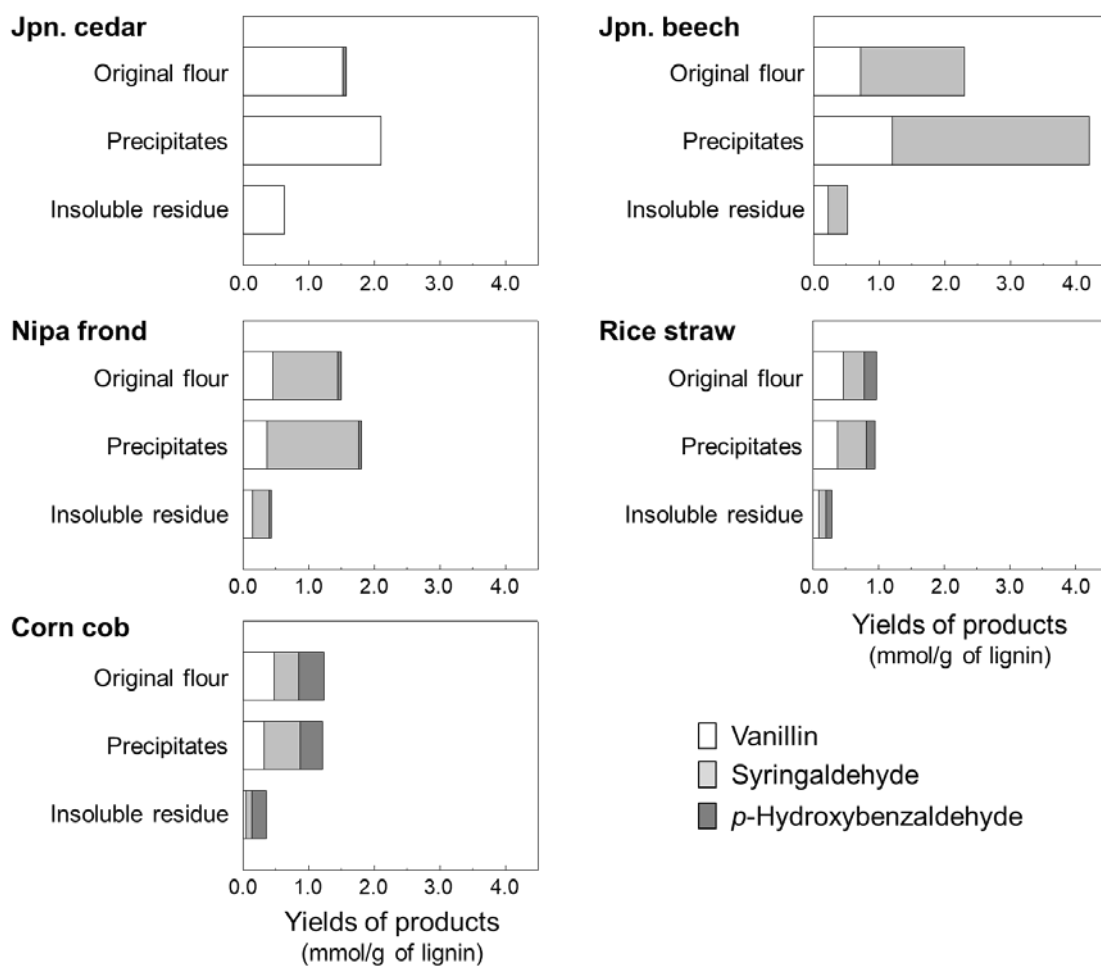

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Fig. 4 GPC chromatographs of lignin-derived products in water-soluble portion and precipitates
from five different lignocellulosics as treated by hot-compressed water

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447 Fig. 5 Yields of alkaline nitrobenzene oxidation products of original flour, precipitates and

448 insoluble residue from five different lignocellulosics as treated by hot-compressed water

449 Table 1 Lignin content, phenolic acids content and alkaline nitrobenzene oxidation yields from five different lignocellulosics

Lignocellulosics	Lignin content (wt%)	Phenolic acids (wt%)			Nitrobenzene oxidation yields (Molar ratio to vanillin)		
		Ferulic acid	p-Coumaric acid	p-Hydroxy benzoic acid	VA*	SA*	HA*
Jpn. Cedar	32.0	-	-	-	1	0	0.05
Jpn. Beech	24.0	-	-	-	1	2.19	0
Nipa frond	19.6	0.13	0.03	0.78	1	2.15	0.10
Rice straw	20.2	0.55	0.79	-	1	0.67	0.41
Corn cob	18.0	0.77	1.50	-	1	0.80	0.79

450   * VA: Vanillin, SA: Syringaldehyde, HA: p-Hydroxybenzaldehyde

451 Table 2 Monomeric compounds in water-soluble portion from five different lignocellulosics as treated by semi-flow hot-compressed water

Lignocellulosics	Lignin-derived monomers (wt%)				Phenolic acids (wt%)		
	Vanillin+ Syringaldehyde	Coniferyl alcohol	Sinapyl alcohol	p-Coumaryl alcohol	Ferulic acid	p-Coumaric acid	p-Hydroxy benzoic acid
Jpn. Cedar	0.06	0.81	-	-	0	-	-
Jpn. Beech	0.20	0.20	0.80	-	0	-	-
Nipa frond	-	0.13	0.26	0	0.01	0.05	0.40
Rice straw	-	0.19	0.01	0.010	0.06	0.18	-
Corn cob	-	0.05	0.14	0.003	0.02	0.52	-

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